## REMARKS

A check for the requisite fee for a three month extension of time accompanies this response. Any fees that may be due in connection with filing this paper or with this application during its entire pendency may be charged to Deposit Account No. 06-1050. If a Petition for extension of time is required, this paper is to be considered such Petition, and any fee charged to Deposit Account No. 06-1050.

Claims 8-14 and 58-72 are pending in this application. Claim 8 is amended to more particularly point out and further clarify the claimed subject matter. As amended, Claim 8 recites that the method of assigning function is a high-throughput method. The high-throughput format is achieved by (1) expressing a plurality of oligonucleotide family members without intervening bacterial cloning steps; and (2) avoiding prior conformational modeling of the target mRNA transcribed from the nucleic acid encoding the product whose function is assigned. The oligonucleotide family members are designed to encode sequences that are complementary to sequences that are distributed throughout the target mRNA.

The claim as amended also clarifies that in the cells where inhibition of production of a product of the target mRNA is observed, a change in phenotype is monitored. The change in phenotype is then analyzed to determine the function associated with a product coded for by a sample nucleic acid sequence in the target nucleic acid molecule.

Claim 58 is amended accordingly to provide proper antecedent basis in Claim 1.

Support for the above amendments are found in the specification, for example, at page 3, lines 13-28; page 4, line 32, through page 5, line 2; page 6, lines 4-22; page 7, lines 7-13; and page 15, lines 3-21. No new matter is added.

Claim 14 is amended herein to clarify that it is phenotypic change that is monitored directly. Basis for this amendment may be found in the specification, for example, at page 15, line 30 to page 16, line 19.

Claims 73 and 74 are added herein. Basis for the added claims are found in the specification, for example, at page 3, line 30 to page 4, line 4; Figures 1A-1C; page 7, lines 14-31; and Example 1 beginning at page 17. No new matter is added.

Attached hereto is a document provided in support of Applicant's arguments below addressing the rejection of Claim 58 and dependents under 35 U.S.C. §112, second paragraph.

**THE REJECTION OF CLAIMS 8-14 AND 58-72 UNDER 35 U.S.C. §112, Second Paragraph**

Claims 8-14 and 58-72 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter. The Office Action provides two bases for the rejections as follows:

First, the Office Action alleges that the metes and bounds of the claims are vague because the purpose of the method states that it is a method of assigning a function corresponding to a phenotype associated with a product, yet the last step recites that the function is assigned based on changes in phenotype. The Examiner alleges that the language of the method appears to require prior knowledge of a particular phenotype associated with a product.

Second, the Office Action alleges that Claims 58 and those dependent therefrom are indefinite in the recitation of the phrase "directionality of expression."

Each of the above two bases are discussed in turn below. Reconsideration of the grounds for rejection is respectfully requested in view of the amendments herein and the following remarks.

**Relevant Law**

Definiteness of claim language must be analyzed, not in a vacuum, but in light of (1) the content of the particular application disclosure, (2) the teachings of prior art, and (3) the interpretation claims would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made. Claims need only "reasonably apprise those skilled in the art" of their scope and be "as precise as the subject permits." *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. den.*, 480 U.S. 947 (1987). The Court in *Orthokinetics, Inc v. Safety Travel Chairs, Inc.*, 1 USPQ2d 1081 (Fed. Cir. 1986) held that a claim limitation requiring that a pediatric wheelchair part be "so dimensioned as to be insertable through the space between the doorframe of an automobile and one of the seats" is definite. The Court stated:

> The phrase 'so dimensioned' is as accurate as the subject matter permits, automobiles being of various sizes. As long as those of ordinary skill in the art realized that the dimensions could be easily obtained, § 112, 2d requires nothing more. The patent law

> does not require that all possible lengths corresponding to the spaces in hundreds of different automobiles be listed in the patent, let alone that they be listed in the claims.

1 USPQ2d at 1088.

When one skilled in the art would understand all of the language in the claims when read in light of the specification, a claim is not indefinite.

Applicant is unaware of any requirement that terms be defined in the claims when one of skill in the art can readily determine the meaning of the term based on the description and definitions provided in the specification. In this respect, applicant is entitled to be its own lexicographer [see, *e.g.*, MPEP 2111.01, "Applicant may be his or her own lexicographer as long as the meaning assigned to the term is not repugnant to the term's well known usage and utilize terms within the claims that are clear from a reading of the specification"]. *In re Hill*, 73 USPQ 482 (CCPA 1947). When applicant has provided definitions in the specification, the claims are interpreted in light of such definition.

35 U.S.C. § 112, second paragraph requires only reasonable precision in delineating the bounds of the claimed invention. The claim language is satisfactory if it reasonably apprises those of skill in the art of the bounds of the claimed invention and is as precise as the subject matter permits. *Shatterproof Glass Corp.v. Libby-Owens Ford Col*, 758 F.2d 613, 624, 225 USPQ 634, 641 (Fed. Cir), *cert dismissed*, 106 S. Ct. 340 (1985).

The amount of detail required to be included in the claims depends on the particular invention and the prior art and is not to be viewed in the abstract, but in conjunction with whether the specification is in compliance with the first paragraph of 35 U.S.C. § 112. If the claims, read in light of the specification, reasonably apprise those skilled in the art of the utilization and scope of the invention, and if the language is as precise as the subject matter permits, the courts can demand no more:

> [i]t is not necessary that a claim recite each and every element needed for the practical utilization of the claimed subject matter (*Bendix Corp. v United States*, 600 F.2d 1364, 1369, 220 Ct. Cl. 507,514, 204 USPQ 617, 621 (1979); *See, also*, *Carl Zeiss Stiftung v. Renishaw plc*, 20 USPQ2d 1094, 1101).

**Analysis**

**1)** It is alleged that the metes and bounds of Claims 8-14 and 58-72 are unclear because the preamble recites a method of assigning a function corresponding to a phenotype associated with a product but the last step states that the function actually is assigned based on changes in phenotype, which allegedly appears to suggest that the phenotype associated with the product already is known. Claim 8 as amended herein no longer recites that the function corresponds to a phenotype. Claim 8 as amended herein that the function is associated with a product and the function is assigned based on an observed change in the host cell phenotype as a result of inhibition of production of the product.

It respectfully is submitted that the metes and bounds of Claim 8 as amended (and claims 9-14 and 58-74, dependent thereon) are clear. As the specification describes in great detail, the claimed method of assigning a function to a product (of interest) encoded by a sample nucleic acid sequence is based on (1) designing an oligonucleotide family library encoding, for example, antisense RNA or ribozymes that contain sequences complementary to mRNA transcribed from a target nucleic acid molecule containing the sample nucleic acid sequence; (2) introducing the oligonucleotide family members into host cells containing the target nucleic acid molecule; (3) expressing the transcription products encoded by the oligonucleotide family members; (4) looking for inhibition of production of a product of the mRNA; and (5) in the host cells that show reduced production of the product, looking for a change in phenotype. **The function of the product of interest is assigned based on the identified phenotypic change** (*see, e.g.*, page 3, lines 26-28; page 6, lines 7-8).

The specification defines "phenotype" as a characteristic of a specific cell or cell population (page 5, lines 3-4). The specification then provides numerous examples of phenotypic characteristics, including, for example: cell viability, replication, morphology, membrane permeability, protein expression, drug susceptibility, *etc.* (page 15, lines 22-27). As the specification describes, all these phenotypic characteristics can first be identified in the host cell by testing or observing each characteristic, then monitoring each identified characteristic for change; no prior knowledge of the whether the phenotype is associated with a product of interest is necessary ((*see, e.g.*, page 15, lines 14-21; page 15, line 30 to page 16, line 6; and page 16, line 11 to page 17, line 2). Further, in the high-throughput format as claimed, many phenotypic

changes can be monitored simultaneously in a plurality of host cells containing the family of oligonucleotides targeting the sample nucleic acid sequence encoding a product of interest (page 3, lines 10-28). The phenotypic changes can be used to assign function to a product of interest without prior knowledge of association of a particular phenotype being associated with the product.

Phenotypic change can be monitored and used to assign function for any and all characteristics regardless of whether the change is one of degree, or the introduction of a new phenotypic characteristic into the host cell. Thus, if inhibition of production of the product of interest initiates apoptosis, certain types of morphologic changes such as nuclear condensation, which was not present in the host cell before treatment with the oligonucleotide family, becomes visible in the treated host cells and the function assigned to the product of interest would be the prevention of apoptosis (page 15, line 31 to page 16, line 1). Other more relative changes such as, for example, decreased cell viability and membrane permeability are detected by differences in staining patterns between treated and untreated host cells; if there is a change, the change is identified as being associated with inhibition of production of a product of the target mRNA and again, no prior knowledge that the product of interest is associated with cell viability or membrane permeability is necessary (page 16, lines 11-13). Any phenotypic characteristic, regardless of prior knowledge of its association with a product of interest, can be monitored and if there is a change in the characteristic, it can be used to assign a function to a product (of interest) encoded by a sample nucleic acid sequence of interest.

Thus, it respectfully is submitted that the metes and bounds of Claim 8 as amended herein are clear: by observing the inhibition of production of a product of the target mRNA in a host cell, then monitoring the resulting host cell for changes in a variety of phenotypic characteristics, regardless of knowledge of a particular phenotype associated with a product, a function can be assigned to a product encoded by a sample nucleic acid sequence contained in a target nucleic acid molecule. Therefore, Claim 8 and Claims 9-14 and 58-72 dependent thereon are not indefinite because when read in light of the specification, one of skill in the art would understand the metes and bounds of the claims.

**2)** It is further alleged that 58-72 are indefinite because the term "directionality" allegedly is not clear. This rejection is respectfully traversed.

It is respectfully submitted that the term "directionality of expression" in the context of cloning expression vectors as recited in claim 58 is a term of art whose meaning was well known as of the application's earliest priority date. For example, in the standard text "Biochemistry" by Garrett and Grisham (Saunders College Publishing, 1995, pp. 251-253, attached hereto), directional cloning is described as the ability to insert the DNA in a particular orientation in a vector so that the gene contained in the DNA molecule is downstream from a promoter and is expressed, *i.e.*, the encoded product is synthezized. The text further describes and pictorially depicts (Fig. 8.6) that directional cloning may be achieved using DNA molecules whose ends have different overhangs.

The specification also describes what is meant by means for determining "directionality of expression" in great detail. For example, at page 6, lines 22-28, the specification describes that directionality may be achieved by incorporating unique restriction sites at each end of the double-stranded DNA to be expressed so that the double-stranded DNA is ligated to the delivery vector "in the correct orientation for expression." The specification further describes, for example, at page 9, lines 7-20 how directionality of expression may be achieved.

Thus, it respectfully is submitted that the metes and bounds of the term "directionality" and the phrase "directionality of expression" are clear in light of the description in the specification and the knowledge of those of skill in the art.

**REJECTION OF CLAIMS 8-14 UNDER 35 U.S.C. §103(a)**

Claims 8-14 are rejected under 35 U.S.C. §103(a) as being unpatentable over Wagner *et al.* U.S. Patent No. 6,355,415 in view of Gudkov *et al.* U.S. Patent No. 5,753,432. It is alleged that it would have been obvious to combine Wagner *et al.*, which allegedly teaches targeting ribozyme constructs that encompass antisense nucleic acid sequences to a sample nucleic acid encoding a product for which a function is to be assigned, then looking for inhibition of production of the product and a corresponding change in phenotype, with Gudkov *et al.*, which allegedly provides specific guidance for amplifying and expressing oligonucleotide constructs without bacterial cloning steps, to arrive at the claimed subject matter. The Examiner further alleges that Gudkov *et al.* "essentially" teaches methods for identifying gene function as the ability of the putative nucleic acid molecules to function as genetic suppressor elements (GSEs)

is unknown prior to testing. This rejection is respectfully traversed. Reconsideration of this rejection is respectfully requested in view of the amendments herein and the following remarks.

**Relevant Law**

In order to set forth a *prima facie* case of obviousness under 35 U.S.C. § 103: (1) there must be some teaching, suggestion or incentive supporting the combination of cited references to produce the claimed invention (ACS Hospital Systems, Inc. v. Montefiore Hospital, 732 F.2d 1572, 1577, 221 USPQ 329, 933 (Fed. Cir. 1984)) and (2) the combination of the cited references must actually teach or suggest the claimed invention. Further, that which is within the capabilities of one skilled in the art is not synonymous with that which is obvious. Ex parte Gerlach, 212 USPQ 471 (Bd. APP. 1980). Obviousness is tested by "what the combined teachings of the references would have suggested to those of ordinary skill in the art." In re Keller, 642 F.2d 413, 425, 208 USPQ 871, 881 (CCPA 1981), but it cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching or suggestion supporting the combination (ACS Hosp. Systems, Inc. v Montefiore Hosp. 732 F.2d 1572, 1577. 221 USPQ 329, 933 (Fed. Cir. 1984)). "To imbue one of ordinary skill in the art with knowledge of the invention in suit, when no prior art reference or references of record convey or suggest that knowledge, is to fall victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher" W.L. Gore & Associates, Inc. v. Garlock Inc., 721 F.2d 1540, 1553, 220 USPQ 303, 312-13 (Fed. Cir. 1983).

Under 35 U.S.C. §103, in order to set forth a case of prima facie obviousness, the differences between the teachings in the cited reference must be evaluated in terms of the whole invention, and the prior art must provide a teaching or suggestion to the person of ordinary skill in the art to have made the changes that would produce the claimed product. See, *e.g.*, Lindemann Maschinen-fabrik Gmbh v. American Hoist and Derrick Co., 730 F.2d 1452, 1462, 221 U.S.P.Q.2d 481, 488 (Fed. Cir. 1984). The mere fact that prior art may be modified to produce the claimed product does not make the modification obvious unless the prior art suggests the desirability of the modification. In re Fritch, 23 U.S.P.Q.2d 1780 (Fed. Cir. 1992); see, also, In re Papesch, 315 F.2d 381, 137 U.S.P.Q. 43 (CCPA 1963). In addition, if the proposed modification or combination of the prior art would change the principle of operation of

the prior art invention being modified, then the teachings of the references are not sufficient to render the claims prima facie obvious. In re Ratti, 270 F.2d 810, 123 USPQ 349 (CCPA 1959).

**THE CLAIMS**

Independent Claim 8 as amended herein is directed to high-throughput method of assigning a function associated with a product coded for by a sample nucleic acid sequence in a target nucleic acid molecule that includes (a) directly delivering and amplifying and expressing as individual transcription products a plurality of members of an oligonucleotide family into a plurality of recombinant non-bacterial host cells, with no prior conformational modeling of the mRNA transcribed from the target nucleic acid molecule and no intervening bacterial cloning steps, where the individual transcription products of the oligonucleotide family members each contain sequences complementary to the mRNA transcribed from the target nucleic acid molecule, the coding sequences for each individual transcription product encodes an antisense RNA that binds to the mRNA transcribed from the target nucleic acid molecule and expression of one or more of the transcription products inhibits production of a product of the mRNA; and (b) in the resulting host cells, analyzing changes in phenotype to thereby assign a function associated with the product encoded by the sample nucleic acid sequence in the target nucleic acid molecule.

Dependent claims further specify types of function, whether phenotypic change is monitored directly, types of sample nucleic acids, numbers of oligonucleotide family members, and types of high-throughput formats. New dependent claims 73 and 74 specify that the oligonucleotide family is a ribozyme family and claim 74 further specifies how the ribozyme oligonucleotide library is designed.

**Differences Between the Claims and the Teachings of the Cited References**

**Wagner *et al.***

Wagner *et al.* is directed to the design of ribozymes that specifically cleave a target nucleic acid sequence of interest in a host cell, inhibit expression of a product encoded by the target nucleic acid and alter a phenotype in the host cell. The altered phenotype is then analyzed to identify the function of the product encoded by the target nucleic acid sequence of interest. Wagner *et al.* teaches that this method may be used to study genes that are homologous to mammalian, including human, genes in suitable model systems such as zebrafish. The function

of the homologous human or mammalian gene can then be deduced by identifying the function of the corresponding gene in zebrafish.

Wagner *et al.* teaches that the ribozymes targeting the nucleic acid sequence of interest are designed by conformational modeling of the secondary structure of the mRNA transcribed from the nucleic acid sequence of interest, identifying substrate cleavage sequences based on the observed secondary structure, then designing ribozymes directed to accessible cleavage sites in the mRNA (col. 17, line 63 to col. 19, line 14). Wagner *et al.* further states that the cleavage sequences may be located anywhere in the mRNA "so long as a ribozyme is capable of cleaving at or near the substrate cleavage site" (col. 19, lines 18-20). Wagner *et al.* further teaches that the binding regions of the ribozymes can be of any length "so long as the desirable specificity of the ribozyme for the RNA substrate and the desirable cleavage rate of the RNA substrate are achieved" (col. 20, lines 47-51). Wagner *et al.* exemplifies this method by demonstrating the computer modeling and identification of 3 accessible sites of mRNA transcribed from zebrafish *ntl* cDNA, then the design of 3 ribozymes that can cleave the 3 accessible sites and their use to identify the function of the *ntl* gene (*see* Example 1 beginning at col. 31).

Wagner *et al.* does not teach or suggest any high-throughput methods of assigning function to a product encoded by a target nucleic acid molecule by constructing an oligonucleotide family library based on complementary sequences throughout the mRNA transcribed from the target molecule of interest. Wagner *et al.* teaches the design of discrete ribozyme molecules directed against a target mRNA by studying the secondary structure of the mRNA for accessible sites. This is a time-consuming operation that is not amenable to a high throughput format. The instant method, on the other hand, avoids the step of assessing secondary structure for sites that are accessible to ribozyme cleavage or to antisense nucleotide binding. Instead, the oligonucleotide family is designed based on sequences that are complementary to sequences throughout the target mRNA. The plurality of oligonucleotide family molecules containing sequences so designed are then introduced into a plurality of host cells, expressed as individual transcription products in the host cells, and the host cells are assessed in high-throughput format for inhibition of expression of the target sequence of interest. Host cells that show inhibition of expression of the target are identified as containing oligonucleotide family members whose transcription products bind to the mRNA molecule

transcribed from the target nucleic acid molecule for whose product a function is assigned. Thus, in the instant methods, the oligonucleotide family contains a plurality of putative antisense or ribozyme sequences, and the high-throughput method screens for the ones that effectively bind to and/or cleave the target mRNA. The oligonucleotide family is not designed based on a discrete selection of molecules that will effectively and/or selectively bind to and/or cleave the target mRNA. Rather, a large number of complementary sequences, regardless of whether their transcription products are effective antisense or ribozyme molecules, are used to make up the oligonucleotide family library.

**Gudkov *et al.***

Gudkov *et al.* is directed to the identification of genetic suppressor elements (GSEs). In the method of Gudkov *et al.*, a random expression library is constructed based on cDNA derived from normal cells. The random library is then screened for inserts that render cells immortalized, tumorigenic or morphologically transformed. The identified GSEs may then be used to screen full length cDNA libraries and identify the corresponding genes.

Gudkov *et al.* does not teach or suggest any method of assigning a function to a product encoded by a known sequence of interest. Gudkov *et al.* identifies heretofore unknown sequences as being GSEs.

The instant method is a method of assigning a function to a known target gene of interest. The target sequence is known, but its function is unknown. The oligonucleotide family used in the instant method is further designed based on this known target sequence (*i.e.*, identifying sequences that are complementary to the known target sequence). The library in Gudkov *et al.*, on the other hand, is a random library of all cDNA, known and unknown sequences, derived from a normal cell. Heretofore unknown (new) GSE sequences are then identified from this random library.

**ANALYSIS**

It is respectfully submitted that the Examiner has failed to set forth a case of *prima facie* obviousness. As discussed above, Wagner *et al.* does not teach or suggest a high-throughput method of assigning a function to a product encoded by a target nucleic acid of interest. In Wagner *et al.*, the ribozymes designed against the mRNA transcribed from the target nucleic acid are discretely identified as being effective at cleaving the mRNA, based on studying the

secondary structure of the mRNA for accessible cleavage sites prior to designing the ribozymes. This is time-consuming and not amenable to a high-throughput screen. The oligonucleotide family library used in the instant high-throughput method, on the other hand, is based on complementary sequences throughout the target mRNA rather than assessing its secondary structure for accessible binding and/or cleavage sites.

Gudkov *et al.*, which identifies new GSEs and is not directed to a method of assigning function to a product encoded by a known sequence of interest, does not cure this deficiency. Moreover, Gudkov *et al.*, which does not teach assigning function to a product encoded by a known sequence, cannot teach design of a library based on sequences that are complementary to the known sequence. The library of Gudkov *et al.* is a random library of fragments derived from cdna from normal cells, from which new GSEs are identified.

There is no teaching or suggestion in either Wagner *et al.* nor Gudkov *et al.*, singly or in combination, of a method of assigning a function to a product encoded by a known target sequence of interest by a high-throughput method in which an oligonucleotide family is designed based on sequences that are complementary to mRNA transcribed from the known target sequence where the mRNA is not assessed for accessible sites in its secondary structure. The instant method screens a plurality of complementary sequences from an oligonucleotide family in a plurality of host cells, then identifies the family members that effectively bind to and/or cleave the target mRNA by identifying those host cells that show inhibition of expression of a product of the mRNA. This renders the method amenable to high-throughput format by avoiding the steps of conformational modeling of secondary structures of the target mRNA and discrete design of ribozyme (or antisense) molecules directed against accessible sites. Neither Wagner *et al.*, which teaches discrete ribozyme design by conformational modeling, nor Gudkov *et al.*, which teaches identification of heretofore unknown sequences possessing a certain property (GSE) from a random cDNA library, singly or in combination, results in the instant high-throughput method. Neither of the cited references, singly or in combination, teaches or suggests the subject matter of the claims. Therefore, the Examiner has failed to set forth a *prima facie* case of obviousness.

* * *

In view of the above amendments and remarks, reconsideration and allowance of the application are respectfully requested.

Respectfully submitted,
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# *Biochemistry*

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tions in the sugar–phosphate backbone of DNA can then be sealed with DNA ligase to yield a covalently closed, circular chimeric plasmid. DNA ligase is an enzyme that covalently links adjacent 3′-OH and 5′-$PO_4$ groups. An inconvenience of this strategy is that *any* pair of *Eco*RI sticky ends can anneal with each other. So, plasmid molecules can reanneal with themselves, as can the foreign DNA restriction fragments. These DNAs can be eliminated by selection schemes designed to identify only those bacteria containing chimeric plasmids.

*Blunt-end ligation* is an alternative method for joining different DNAs. This method depends on the ability of **phage T4 DNA ligase** to covalently join the ends of any two DNA molecules (even those lacking 3′- or 5′-overhangs) (Figure 8.4). Some restriction endonucleases cut DNA so that blunt ends are formed (see Table 6.5). Since there is no control over which pair of DNAs are blunt-end ligated by T4 DNA ligase, strategies to identify the desired products must be applied.

A great number of variations on these basic themes have emerged. For example, short synthetic DNA duplexes whose nucleotide sequence consists of little more than a restriction site can be blunt-end ligated onto any DNA. These short DNAs are known as **linkers.** Cleavage of the ligated DNA with the restriction enzyme then leaves tailor-made sticky ends useful in cloning reactions (Figure 8.5). Similarly, many vectors contain a **polylinker** cloning site, a short region of DNA sequence bearing numerous restriction sites.

### *Promoters and Directional Cloning*

Note that the strategies discussed thus far create hybrids in which the orientation of the DNA insert within the chimera is random. Sometimes it is desirable to insert the DNA in a particular orientation. For example, an experimenter might wish to insert a particular DNA (a gene) in a vector so that its

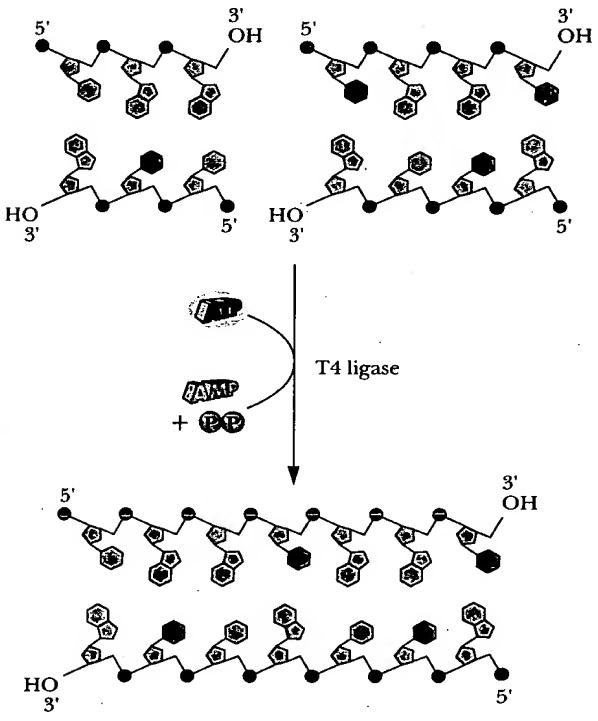
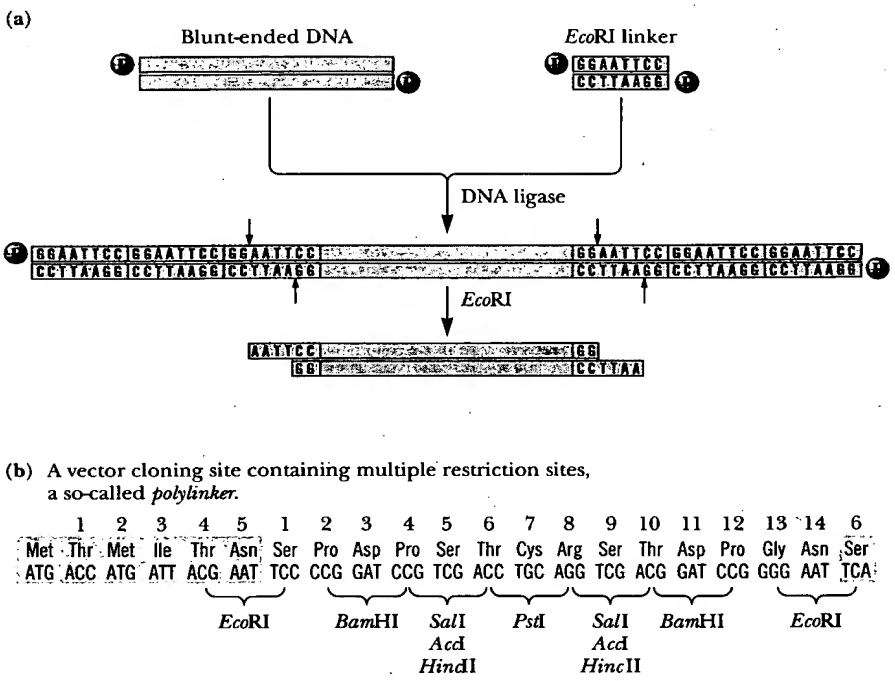



*Figure* 8.4 Blunt-end ligation using phage T4 DNA ligase, which catal the ATP-dependent ligation of DNA molecules. AMP and $PP_i$ are by-products.




(b) A vector cloning site containing multiple restriction sites, a so-called *polylinker.*

| 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Met | Thr | Met | Ile | Thr | Asn | Ser | Pro | Asp | Pro | Ser | Thr | Cys | Arg | Ser | Thr | Asp | Pro | Gly | Asn | Ser |
| ATG | ACC | ATG | ATT | ACG | AAT | TCC | CCG | GAT | CCG | TCG | ACC | TGC | AGG | TCG | ACG | GAT | CCG | GGG | AAT | TCA |
| | | | | *EcoRI* | | | *BamHI* | | *SalI* *AccI* *HindII* | | *PstI* | | *SalI* *AccI* *HincII* | | *BamHI* | | | *EcoRI* | | |

***Figure* 8.5** (a) The use of linkers to create tailor-made ends on cloning fragments. Synthetic oligonucleotide duplexes whose sequences represent *Eco*RI restriction sites are blunt-end ligated to a DNA molecule using T4 DNA ligase. Note that the ligation reaction can add multiple linkers on each end of the blunt-ended DNA. *Eco*RI digestion removes all but the terminal one, leaving the desired 5′-overhangs. (b) Cloning vectors often have polylinkers consisting of a multiple array of restriction sites at their cloning sites, so restriction fragments generated by a variety of endonucleases can be incorporated into the vector. Note that the polylinker is engineered not only to have multiple restriction sites but also to have an uninterrupted sequence of codons, so this region of the vector has the potential for translation into protein. The sequence shown is the cloning site for the vectors M13mp7 and pUC7; the colored amino acid residues are contiguous with the coding sequence of the *lacZ* gene carried by this vector (see Figure 8.19).
*(a, Adapted from Figure 3.16.3; b, adapted from Figure 1.14.2, Ausubel, F. M., et al., 1987, Current Protocols in Molecular Biology. New York: John Wiley and Sons.)*

gene product is synthesized. To do this, the DNA must be placed downstream from a **promoter.** A promoter is a nucleotide sequence lying upstream of a gene that is involved in regulating expression of the gene. RNA polymerase molecules bind specifically at promoters and initiate transcription of adjacent genes, copying template DNA into RNA products. One way to insert DNA so that it will be properly oriented with respect to the promoter is to create DNA molecules whose ends have different overhangs. Ligation of such molecules into the plasmid vector can only take place in one orientation, called **directional cloning** (Figure 8.6).

### *Biologically Functional Chimeric Plasmids*

The first biologically functional chimeric DNA molecules constructed *in vitro* were assembled from parts of different plasmids in 1973 by Stanley Cohen, Annie Chang, Herbert Boyer, and Robert Helling. These plasmids were used to **transform** recipient *E. coli* cells (*transformation* means the uptake and replication of exogenous DNA by a recipient cell; see Chapter 28). The bacterial

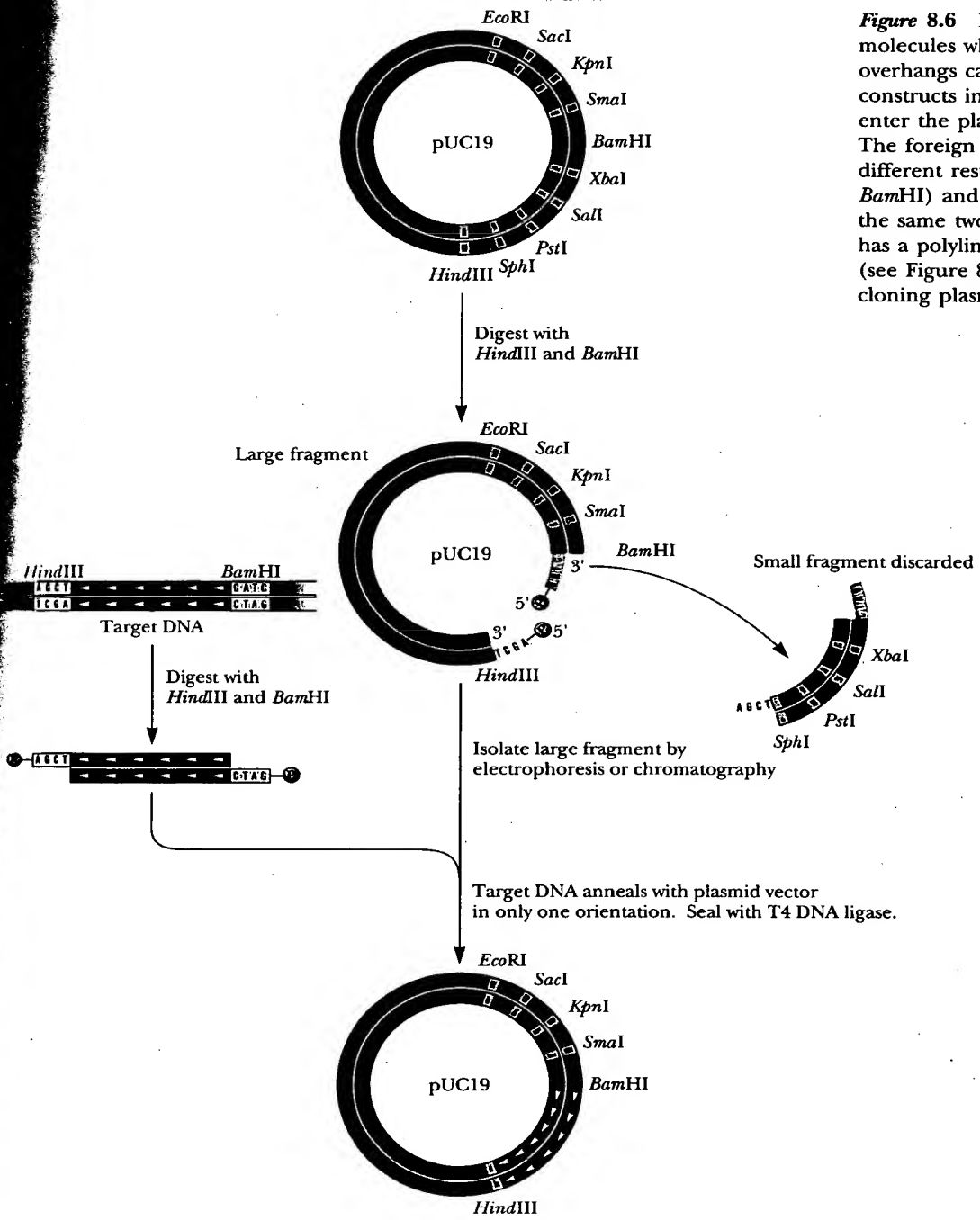



***Figure* 8.6** Directional cloning. DNA molecules whose ends have different overhangs can be used to form chimeric constructs in which the foreign DNA can enter the plasmid in only one orientation. The foreign DNA is digested with two different restriction enzymes (*Hin*dIII and *Bam*HI) and the plasmid is digested with the same two enzymes. Note that pUC19 has a polylinker or universal cloning site (see Figure 8.5b); pUC stands for universal cloning plasmid.

cells were rendered somewhat permeable to DNA by $Ca^{2+}$ treatment and a brief 42°C heat shock. Although less than 0.1% of the $Ca^{2+}$-treated bacteria became competent for transformation following such treatment, transformed bacteria could be selected by their resistance to certain antibiotics (Figure 8.7). Consequently, the chimeric plasmids must have been biologically functional in at least two aspects: they replicated stably within their hosts and they expressed the drug resistance markers they carried.

In general, plasmids used as cloning vectors are engineered to be small, 2.5 kbp to about 10 kbp in size, so that the size of the insert DNA can be